Multiway calibration strategy with chromatographic data exploiting the second-order advantage for quantitation of three antidiabetic and three antihypertensive drugs in serum samples

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ABSTRACT

This paper proposes a multiway calibration strategy implementing the modeling with MCR-ALS and U-PLS/RBL of second-order chromatographic data for quantitation of six analytes: gliclazide, glibenclamide, glimepiride, atenolol, enalapril and amlodipine in serum samples, in an analysis time of 3 min.
The performance of both algorithms was compared in terms of predictive ability, showing relative error of prediction values below 10% in all cases. LOD values calculated are below 30 ng mL^{-1} for all the studied drugs, which allow detection in human serum in patients under treatment. U-PLS/RBL has higher sensitivity and better detection and quantification limits for all the studied analytes; however results obtained by MCR-ALS enable its usage as well. Both methods provide comparable results for glibenclamide, glimepiride and gliclazide. With this multiway calibration strategy, the presence of enalapril, amlodipine and atenolol could be quantitated with high accuracy. Run time was reduced by 50% considering previous reports, as well as reduction of solvents, in accordance with green chemistry principles.

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1. Introduction

Diabetes is a disease affecting 9% of the Argentinian population [1]. Type II diabetes is the most common form of diabetes in which cellular resistance to insulin and insufficient pancreatic secretion derive in hyperglycemia. In order to diagnose and treat type II diabetes properly, it is mandatory to develop analytical methods for management and pharmacological treatment monitoring.

Sulfonylureas are oral antidiabetic drugs that increase insulin release form pancreatic beta cells. Gliclazide, glibenclamide and glimepiride are second generation sulfonylurea drugs used as initial treatment of type II diabetes in patients who cannot control hyperglycemia with diet and exercise [2].

Diabetic patients also have a high prevalence of hypertension. Pharmacological therapy frequently combines antihypertensive and antidiabetic drugs [3]. Atenolol belongs to the beta blocker drug group; enalapril is an angiotensin-converting enzyme inhibitor; and amlodipine is a calcium channel blocker, all of these with antihypertensive action. Usual seric concentration of the three antihypertensive and the three antidiabetic analyzed drugs are: atenolol 0.30–0.70 μg mL^{-1}; amlodipine 0.004–0.017 μg mL^{-1}; enalapril 0.l5–0.30 μg mL^{-1}; gliclazide 2.00–8.00 μg mL^{-1}; glibenclamide 0.14–0.35 μg mL^{-1}; and glimepiride 0.20–0.31 μg mL^{-1} [4].

In a previous work, we developed a novel dispersive liquid–liquid micro extraction (DLLME) procedure and a HPLC–UV method, optimized and fully validated for the determination of gliclazide, glibenclamide and glimepiride in serum, in the presence of atenolol, enalapril and amlodipine. The advantages of the latter method are simplicity of operation, rapidity, low cost, high–recovery, high enrichment factor, and environmental benignity fitting the requirements of green analytical chemistry [5].

Multivariate calibration strategies are quickly gaining attention in analytical chemistry, given the possibility of quantifying analytes in complex matrixes in the presence of interferents. If one calibrates with pure analyte standards, matrix data is recorded and sufficient selectivity is present in the various data modes; it is possible to predict analyte concentration in any future sample, no matter how many signal-overlapping constituents. This is referred to as the "second order advantage", the signal from unexpected constituents can be modeled and mathematically removed, in such a way that their effect is negligible [6].

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An important number of algorithms have been used in order to exploit the second-order advantage: generalized rank annihilation (GRAM) [7], direct trilinear decomposition (DTLD) [8], self-weighted alternating trilinear decomposition (SWATLD) [9], alternating penalty trilinear decomposition (APTLD) [10], parallel factor analysis (PARAFAC) [11], multivariate curve resolution alternating least squares (MCR–ALS) [12], bilinear least squares (BLLS) [13], unfolded partial least squares/residual bilinearization (U–PLS/RBL) [14] and artificial neural networks followed by residual bilinearization (ANN/RBL) [15].

In this paper, a multiway calibration strategy implementing two well-known algorithms (MCR-ALS and U-PLS/RBL) are proposed for quantitation of gliclazide, glibenclamide, glimepiride, atenolol, enalapril and amlodipine in serum samples, in a shorter analysis time than that previously reported [5].

2. Theory

2.1. MCR-ALS

This algorithm works on a data set by optimizing initial estimates in an ALS way within each iterative cycle under the action of suitable constraints until a convergence criterion is fulfilled [16].

Each HPLC-DAD chromatographic run of a single sample provides a data matrix, indicated as $\mathbf{D}$ ($J \times K$), where the J rows representing the UV spectra recorded at the different elution times and the K columns representing the chromatographic elution profiles recorded at the different wavelengths. This corresponds to a bilinear model based on the multi wavelength extension of Beer's absorption law:

$$\mathbf{D} = \mathbf{CS}^{\mathrm{T}} + \mathbf{E} \tag{1}$$

where $\mathbf{C}$ ($J \times N$) is the matrix of elution profiles of the analyzed compounds and $\mathbf{S}^{\mathrm{T}}$ ($N \times K$) is the matrix of their pure spectra. N is the number of components.

Multiset structures are obtained combining several chromatographic runs. These structures are organized appending the $\mathbf{D}_i$ data matrices (the index i indicates a chromatographic run for a specific sample) one on top of each other. The resulting $\mathbf{D}_{\mathrm{aug}}$ (column-wise augmented) multiset can be decomposed into the $\mathbf{C}_{\mathrm{aug}}$ matrix, which contains the $\mathbf{C}_i$ submatrices of the resolved elution profiles for the single chromatographic runs, $\mathbf{S}^{\mathrm{T}}$ the matrix of pure spectra common to all chromatograms analyzed and $\mathbf{E}_{\mathrm{aug}}$, the difference between the raw data and the reconstructed data by the $\mathbf{C}_{\mathrm{aug}}\mathbf{S}^{\mathrm{T}}$ model, i.e., the experimental error not explained by the bilinear model. This bilinear model assumes that the components in the $\mathbf{D}_i$ data matrices included in the column-wise augmented data matrix share the same pure spectra, whereas they can have different concentration profiles.

The MCR-ALS algorithm calculates $\mathbf{C}_{\mathrm{aug}}$ and $\mathbf{S}^{\mathrm{T}}$ from the sole information in the experimental data, $\mathbf{D}_{\mathrm{aug}}$. The first step is to determine the number of eluted compounds present in a particular cluster of peaks, i.e., the "chemical rank" associated with the data matrix. This determination is performed with a principal component analysis on the $\mathbf{D}_{\mathrm{aug}}$ matrix. Then, an initial estimate of the $\mathbf{S}^{\mathrm{T}}$ matrix is obtained with techniques based on the detection of purest variables [17]. These initial spectral estimates are iteratively optimized with a constrained alternating least squares regression procedure.

The iterative optimization is performed until the results agree with the convergence criterion, which often means that the difference in lack of fit between two consecutive iterations is below a predefined threshold (0.01% change in standard deviation). The lack of fit (%LOF) and the explained variance (EV) express the fitting quality of the resolution results; they are used to choose the best MCR-ALS model for each chromatographic segment.

Several constraints can be applied to confer chemical meaning to the profiles obtained by MCR-ALS in the analysis of a single HPLC-DAD run, such as non-negativity, unimodality, spectral normalization and component correspondence in order to reduce the effects of rotational ambiguity.

Finally, the areas under the matrix $\mathbf{D}_{\mathrm{aug}}$ are used to build a pseudounivariate plot relating them with the nominal concentrations of the standards. This plot is then used to predict the analyte concentration in the unknown sample.

2.2. U-PLS/RBL

Unfolded partial least-squares (U–PLS) is a powerful algorithm for processing vectorial signals per sample, it provides multiway data processing with enough flexibility to face calibration protocols based on complex data [16]. It is complemented by residual bilinearization (RBL) which models the residues of U-PLS for the test sample as a sum of bilinear contributions from the unexpected components.

The first step in U-PLS calibration is to convert the calibration data arrays into vectors. This will produce a $JK \times 1$ vector from a $J \times K$ data matrix. A new calibration matrix $\mathbf{X}_{\mathrm{cal}}$, suitable for the application of PLS regression, is built by placing all column vectors adjacent to each other. The latter $\mathbf{X}_{\mathrm{cal}}$ matrix can therefore be of size $JK \times I$ ($I =$ number of calibration samples) for second-order data, and is subjected to the classical PLS regression analysis.

As it is well-known for PLS, a set of loadings $\mathbf{P}$ and weight loadings $\mathbf{W}$ ($JK \times A$, where A is the number of latent variables) as well as regression coefficients $\mathbf{v}$ (size $A \times 1$) are obtained after the calibration step. Usually, the leave-one-out cross-validation procedure is implemented for selecting the parameter A [18]. Subsequently, $\mathbf{v}$ is employed to estimate the analyte concentration through the following equation:

$$y_{\mathrm{u}} = \mathbf{t}_{\mathrm{u}}^{\mathrm{T}}\mathbf{v} \tag{2}$$

where $\mathbf{t}_{\mathrm{u}}$ (size $A \times 1$) is the test sample score, obtained by projection of the (unfolded) data for the test sample $\mathbf{X}_{\mathrm{u}}$ [vec($\mathbf{X}_{\mathrm{u}}$)] of size ($JK \times 1$) onto the space of the A latent factors:

$$\mathbf{t}_{\mathrm{u}} = \left(\mathbf{W}^{\mathrm{T}}\mathbf{P}\right)^{-1}\mathbf{W}^{\mathrm{T}}\mathrm{vec}(\mathbf{X}_{\mathrm{u}}) \tag{3}$$

If the sample contains unexpected components, the scores given by Eq. (3) are not suitable for analyte prediction using Eq. (2), generating abnormally large residuals in comparison with the typical instrumental noise assessed by replicate measurements.

RBL intends to model the residuals assuming that they can be arranged into a bilinear matrix. This procedure fits the sample data to the sum of two contributions: 1) the portion of the test data, which can be explained by the calibration PLS loadings, and 2) the contribution from the potential interferents modeled by a certain number of principal components (N_{RBL}). The complete U-PLS/RBL modeling equation involves a residual error term to be minimized by least squares:

$$\mathbf{X}u = \mathrm{reshape}(\mathbf{Pt}_{\mathrm{RBL}}) + \mathbf{B}_{\mathrm{RBL}}\mathbf{T}_{\mathrm{RBL}}^{\mathrm{T}} + \mathbf{E}_{\mathrm{RBL}} \tag{4}$$

The product ($\mathbf{B}_{\mathrm{RBL}}\mathbf{T}_{\mathrm{RBL}}^{\mathrm{T}}$) is the principal component analysis (PCA) model for the residual matrix [($\mathbf{X}u$–reshape($\mathbf{Pt}_{\mathrm{RBL}}$)] with N_{RBL} principal components, with "reshape" meaning the transformation $JK \times 1$ vector into a $J \times K$ data matrix. Minimization of $\mathbf{E}_{\mathrm{RBL}}$ allows one to retrieve the final score vector $\mathbf{t}_{\mathrm{RBL}}$. Initially, the residual matrix contains contributions from both the calibrated analytes and the potential interferents. Modeling this latter matrix with PCA extracts N_{RBL} bilinear components; the more these bilinear components resemble the unexpected contributions, the better the product is able to model the behavior of the analytes in the test sample, leading to a continuous decrease in the residuals.

Generally RBL can be carried out by a Gauss-Newton minimization. Once the residuals $\mathbf{E}_{\mathrm{RBL}}$ are minimized in Eq. (4), the output is a final $\mathbf{t}_{\mathrm{RBL}}$ vector which represents the true contribution of the calibrated

analytes to the test sample. Analyte concentrations are the obtained by introducing the final $\mathbf{t}_{RBL}$ vector instead of t in Eq. (3). While calibration data are unfolded to be processed by U-PLS with greater flexibility, test data should be maintained in matrix form in order to be able to apply PCA within the RBL procedure.

3. Experimental

3.1. Apparatus and software

All experiments were performed using an Agilent 1100 Series liquid chromatograph equipped with a quaternary pump, degasser membrane, thermostated column compartment, auto sampler and DAD (Agilent Technologies, Waldbronn, Germany). Multiwavelength information was registered every 2 nm between 200 and 400 nm. The Chemstation version B 0103 was used for data acquisition and processing. The HPLC column was a Zorbax C18 (4.6 mm × 75 mm, 3.5 μm particle size) from Agilent.

3.2. Chemicals and reagents

Analytical standards of atenolol, amlodipine, enalapril and glimepiride were provided by PLAMECOR (Medicinal Plant of Corrientes, Argentina). Glibenclamide and gliclazide were provided by Roemmers Argentina (Buenos Aires, Argentina). Acetonitrile and methanol HPLC-grade were obtained from Merck (Darmstadt, Germany). HPLC-grade water was obtained from a Milli-Q Biocel System (Millipore SAS, Molsheim, France). Sodium hydroxide, dichloromethane and monosodium phosphate of analytical grade were purchased from Cicarelli (San Lorenzo, Argentina).

Solutions and solvents for mobile phase were always filtered through 0.45 μm nylon filters. Standards and sample solutions were also filtered through syringe 0.45 μm nylon membrane before injection in the chromatographic system. Photometric detection was performed in the 200–400 nm range.

3.3. Chromatographic conditions

The chromatographic conditions were set based on previous knowledge about the system [5]. In this work, however, the main objective was to reduce the run time and, consequently, the amount of waste solvents. Thus, the mobile phase (isocratic mode) was 37:63 acetonitrile:sodium phosphate buffer 10 mmol L^{-1} (pH 2.6). Column temperature was controlled by setting the oven temperature at 30 °C and the flow rate was maintained at 1.00 mL min^{-1}. An injection volume of 20 μL was used. The complete analysis was carried out in 3 min.

3.4. Standard solutions

Stock standard solutions of atenolol, amlodipine and enalapril 1.0 mg mL^{-1} were prepared by exactly weighing and dissolving the adequate amount of standard in ultrapure water. Glibenclamide stock solution 1.0 mg mL^{-1} was prepared by weighing and dissolving the adequate amount of standard in acetonitrile. Gliclazide stock solution 1.0 mg mL^{-1} was prepared by weighing and dissolving the adequate amount of standard in methanol. Glimepiride stock solution 1.0 mg mL^{-1} was prepared by weighing and dissolving the adequate amount of standard in NaOH 0.1 mol L^{-1}. These solutions were conserved at 4 °C in light-resistant containers and allowed to reach room temperature before use. Calibration standard solutions were prepared at the moment of use by diluting an appropriate volume of each stock standard solution in ultrapure water.

3.5. Sample preparation

In a previous work, we developed a novel dispersive liquid-liquid micro extraction pre-treatment, which was followed herein [5]. Plasma sample or standard solutions aliquots of 250 μL were transferred into 1.5 mL centrifuge tubes, 100 μL of dichloromethane (extractive solvent) and 1000 μL of acetonitrile (dispersing solvent) were added. Finally, the samples were vortexed for 1 min, centrifuged at 1000 g for 3 min and the organic phase was transferred to glass tubes. The organic phase was finally evaporated to dryness under a gentle stream of nitrogen gas. The residue was dissolved in 50 μL acetonitrile:sodium phosphate buffer 10 mmol L^{-1} pH 2.6 mixture (50:50) and injected into the HPLC system. Consequently, an enrichment factor of 5 was reached with the pre-treatment.

3.6. Calibration and validation samples

In our previous work [5] it was demonstrated that there was no matrix effect, therefore, calibration and validation sets were prepared in ultrapure water. A calibration set of 14 samples that contained the 6 studied drugs was prepared from the diluted solutions in ultrapure water. The samples of the set corresponded to the concentrations provided by a central composite design, obtained by combining two central composite designs of three drugs each, therefore the set contained only 14 samples. The tested concentrations were in the range of 0.06–2.60 μg mL^{-1} for gliclazide; 0.03–1.92 μg mL^{-1} for glibenclamide; 0.04–1.98 μg mL^{-1} for glimepiride; 0.15–2.75 μg mL^{-1} for atenolol; 0.08–2.82 μg mL^{-1} for enalapril and 0.06–2.80 μg mL^{-1} for amlodipine.

A validation set of 11 samples that contained the 6 studied drugs was prepared from the diluted solutions in ultrapure water. The samples of the latter set corresponded to concentrations provided by a central composite design. The tested concentrations were in the range of 0.08–1.94 μg mL^{-1} for gliclazide; 0.09–1.56 μg mL^{-1} for glibenclamide; 0.13–1.40 μg mL^{-1} for glimepiride; 0.65–2.61 μg mL^{-1} for atenolol; 0.5–1.72 μg mL^{-1} for enalapril and 0.14–2.52 μg mL^{-1} for amlodipine (Table 1).

HPLC-DAD matrix data were obtained and subjected to second-order data analysis.

3.7. Precision and trueness

Recovery was assessed by replicate analysis ($n = 3$) of spiked samples at three different concentrations (Table 2), prepared by spiking blank human plasma with a convenient volume of standard solution. The samples of the set corresponded to the concentrations provided by a central composite design. Repeatability was assessed by replicate analysis of the recovery samples. Relative standard deviation (RSD %) was calculated.

3.8. Data generation and software

Every data matrix contains 195 rows (elution times) and 101 columns (wavelengths). The total chromatographic data registered for each sample were partitioned in 2 regions: a) from 0.38 to 1.15 min and 200 to 400 nm (R1 corresponding to antihypertensive drugs), and b) from 1.22 to 2.18 min 202 to 360 nm (R2 corresponding to antidiabetic drugs). These regions are shown in Fig. 1. It should be noted that this was done in order to reduce using non informative signals.

All employed algorithms were implemented in MATLAB 7.10.0.499 [19] A useful interface for data input and parameters setting, written by Olivieri et al. [20] was employed for MCR-ALS and U-PLS/RBL implementation.

Table 1
Predictions on validation samples by modeling second-order data.

Validation sample	Atenolol			Enalapril			Amlodipine			Gliclazide			Glibenclamide			Glimepiride		
	Nom	U-PLS/RBL Pred	MCR-ALS Pred	Nom	U-PLS/RBL Pred	MCR-ALS Pred	Nom	U-PLS/RBL Pred	MCR-ALS Pred	Nom	U-PLS/RBL Pred	MCR-ALS Pred	Nom	U-PLS/RBL Pred	MCR-ALS Pred	Nom	U-PLS/RBL Pred	MCR-ALS Pred
1	2.01	1.97	1.96	1.41	1.45	1.35	1.45	1.46	1.57	1.19	1.25	1.17	1.47	1.42	1.46	0.84	0.76	0.77
2	1.83	1.83	1.92	1.72	1.73	1.63	1.00	0.99	0.94	1.94	2.00	2.06	1.15	1.16	1.18	0.84	0.88	0.90
3	0.65	0.67	0.66	0.88	0.85	0.80	0.14	0.14	0.13	1.13	1.14	1.16	1.18	1.17	1.27	1.40	1.38	1.51
4	1.40	1.42	1.38	0.50	0.51	0.46	1.18	1.16	1.13	1.13	1.08	1.11	1.18	1.19	1.21	1.40	1.33	1.45
5	1.83	1.84	1.86	0.88	0.92	0.83	1.73	1.78	1.83	1.84	1.83	1.67	0.12	0.15	0.11	1.40	1.35	1.35
6	1.02	1.05	1.07	0.65	0.66	0.64	1.73	1.74	1.74	1.30	1.36	1.33	0.09	0.10	0.09	0.54	0.60	0.62
7	1.77	1.78	1.62	1.72	1.62	1.73	0.84	0.84	0.78	1.92	2.12	1.98	1.56	1.57	1.64	0.13	0.13	0.14
8	2.01	1.94	2.04	1.05	1.01	1.05	1.45	1.35	1.43	1.19	1.18	1.04	1.47	1.48	1.50	1.20	1.12	1.15
9	1.02	1.07	0.92	1.05	0.98	1.09	1.00	1.13	0.93	0.08	0.08	0.07	1.47	1.46	1.52	1.40	1.34	1.45
10	1.45	1.31	1.42	0.50	0.47	0.54	1.00	1.02	1.13	1.19	1.10	1.18	0.92	0.99	0.95	0.84	0.89	0.80
11	2.01	1.84	2.17	1.41	1.37	1.56	2.52	2.66	2.36	0.08	0.10	0.07	0.18	0.16	0.18	0.16	0.14	0.17
RMSE[a]		0.07	0.08		0.05	0.07		0.07	0.09		0.08	0.08		0.03	0.04		0.05	0.06
REP%[b]		4.76	5.32		4.31	6.18		5.28	6.77		6.36	6.87		2.98	4.43		5.87	6.44
Mean recovery ($\overline{R}_{exp}$)		99.1	99.8		98.5	99.0		101.4	98.9		103.2	96.6		102.8	101.6		98.1	102.7
Standard deviation of recovery (SR)		4.7	5.5		4.0	6.4		4.9	7.1		8.7	7.1		9.3	4.1		7.0	7.0
t_{exp}[c]		0.66	0.10		1.29	0.51		0.92	0.49		1.24	1.57		1.00	1.30		0.91	1.29

Nom: nominal concentration ($\mu g\ mL^{-1}$) and Pred: predicted concentration ($\mu g\ mL^{-1}$).

[a] RMSE: Root mean square error, $RMSE = \sqrt{\frac{1}{I}\sum_{1}^{I}(c_{nom}-c_{pred})^2}$, where $I = 11$.

[b] REP: Relative error of prediction, $REP = 100 \times \frac{RMSE}{\overline{c}}$, where $\overline{c}$ is the mean calibration concentration.

[c] Experimental t_{exp} value, $t_{exp} = |100-\overline{R}_{exp}|\frac{\sqrt{N}}{S_R}$, where $N = 11$ is the number of validation samples. (Critical value $t_{(0.025,10)} = 2.228$).

Table 2
Recovery study in spiked basal plasma[a].

Sample	Atenolol			Enalapril			Amlodipine			Gliclazide			Glibenclamide			Glimepiride		
	Taken	U-PLS/RBL Found	MCR-ALS Found	Taken	U-PLS/RBL Found	MCR-ALS Found	Taken	U-PLS/RBL Found	MCR-ALS Found	Taken	U-PLS/RBL Found	MCR-ALS Found	Taken	U-PLS/RBL Found	MCR-ALS Found	Taken	U-PLS/RBL Found	MCR-ALS Found
T1_1	0.61	0.61	0.63	0.50	0.51	0.46	0.45	0.48	0.43	0.23	0.25	0.22	0.33	0.35	0.34	1.07	1.02	1.02
T1_2	0.61	0.62	0.61	0.50	0.50	0.51	0.45	0.47	0.48	0.23	0.24	0.21	0.33	0.33	0.32	1.07	1.05	1.05
T1_3	0.61	0.60	0.62	0.50	0.53	0.48	0.45	0.44	0.45	0.23	0.22	0.21	0.33	0.34	0.33	1.07	1.02	1.06
T2_1	0.80	0.86	0.76	1.41	1.47	1.35	1.92	1.94	1.84	1.32	1.36	1.29	0.17	0.17	0.16	0.63	0.63	0.60
T2_2	0.80	0.83	0.78	1.41	1.39	1.38	1.92	1.89	1.92	1.32	1.36	1.26	0.17	0.18	0.16	0.63	0.67	0.64
T2_3	0.80	0.85	0.79	1.41	1.42	1.42	1.92	1.91	1.85	1.32	1.35	1.25	0.17	0.16	0.17	0.63	0.62	0.62
T3_1	0.51	0.49	0.52	0.95	0.92	0.97	0.30	0.31	0.32	1.20	1.16	1.24	0.25	0.23	0.24	0.10	0.11	0.10
T3_2	0.51	0.52	0.51	0.95	0.95	0.94	0.30	0.30	0.31	1.20	1.17	1.20	0.25	0.24	0.25	0.10	0.10	0.10
T3_3	0.51	0.51	0.49	0.95	0.93	0.93	0.30	0.29	0.29	1.20	1.19	1.22	0.25	0.24	0.24	0.10	0.11	0.10
Mean recovery ($\overline{R}_{exp}$)		101.7	99.4		100.7	98.1		100.9	100.1		101.2	96.8		99.2	97.8		101.5	98.4
Standard deviation of recovery (S_R)		3.7	2.8		3.0	3.3		3.3	4.5		4.2	4.3		5.1	3.1		5.8	2.1
t_{exp}[b]		1.42	0.70		0.70	1.70		0.79	0.08		0.82	2.26		0.45	2.14		0.78	2.26

[a] Concentration are given in $\mu g\ mL^{-1}$. Recovery (between parentheses) is given in percentage.

[b] Experimental t_{exp} value, $t_{exp} = |100-\overline{R}_{exp}|\frac{\sqrt{N}}{S_R}$, where $N = 9$ is the number of validation samples. (Critical value $t_{(0.025,8)} = 2.306$).
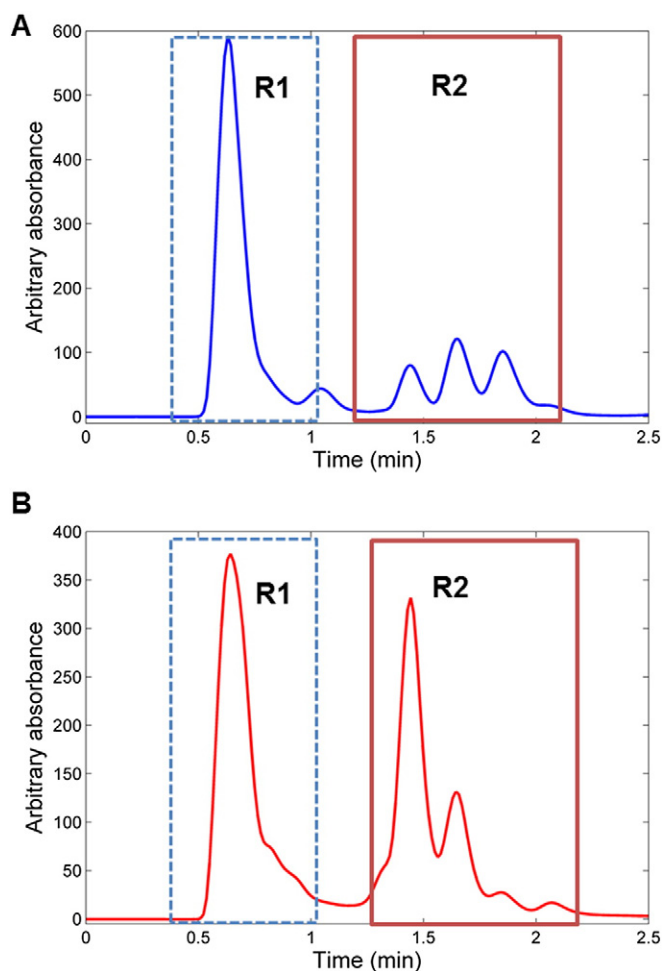
Fig. 1. Chromatographic runs corresponding to a standard solution prepared in water (A) and basal serum (B), registered at 230 nm. The total chromatographic data registered for each sample were partitioned in: Region 1 (R1) corresponding to antihypertensive drugs (from 0.38 to 1.15 min and 200 to 400 nm); and Region 2 (R2) corresponding to antidiabetic drugs (from 1.22 to 2.18 min and 202 to 360 nm).

4. Results and discussion

4.1. Multiway analysis

In our previous work [5], we could only quantitate hyperglycemic drugs, while antihypertensive drugs appeared overlapping matrix components. This problem has been solved in the present report by the use of three-way modeling.

Before MCR-ALS and U-PLS/RBL processing, the chromatographic data were split into two regions, R1 and R2 (see above). Fig. 1 shows the chromatographic runs corresponding to a standard solution prepared in water (Fig. 1A) and basal serum (Fig. 1B), both registered at 230 nm.

4.2. MCR-ALS and U-PLS/RBL implementation

In order to quantitate the six analyzed drugs in the 11 validation samples (Table 1), the number of contributions to each **D** data matrix was determined by singular value decomposition (SVD). Taking into account that MCR-ALS needs information as real as possible to start the resolution, the S^T–type initial estimated were built combining the pure analyte chromatograms with those obtained for the interferences by means of the selection of purest time profiles based on estimation of purest variables [17]. Given **D** and S^T, appropriate constraints

(i.e. non–negativity and unimodality) were implemented to drive the iterative optimization to the right solution. After MCR-ALS of **D** according to Eq. (1), the concentration information contained in **C** was used to construct the univariate graph by plotting the analyte concentration scores against the nominal analyte concentrations. The analyte concentration score can be defined as the area under the profile for the sample:

$$a(i,n) = \sum_{j=1+(i-1)J}^{iJ} C(j,n) \tag{5}$$

in which $a(i,n)$ is the score for the component n in the sample i.

To assess the quality of the modeling, the degree of rotational ambiguity was evaluated in order to know if the solutions provided by the decomposition are practically unique. For this purpose, the software presented by Jaumot and Tauler was implemented [21]. Table 3 shows the parameters f^{min} and f^{max} and its corresponding difference obtained when the available constraints were imposed. Modeling was performed implementing the constraints normalization, non-negativity, unimodality and trilinearity (the latter one with bad results). It showed that profiles do not change during the optimization. This conclusion is reached by analyzing the relationships between f^{max} and f^{min} which should be nearest to zero (Table 3). As can be appreciated, the data do not follow the trilinearity property owing to when this constraint is applied worst results are obtained (fifth column of Table 3).

UPL-S/RBL was applied first selecting the number of latent variables by the well-known leave-one sample-out cross-validation procedure [18]. Thus, the optimum number of factors was estimated by calculating the ratios:

$$F(A) = PRESS(A<A^*)/PRESS(A) \tag{6}$$

where $PRESS = \sum (y_{i,\ act} - y_{i,pred})^2$, A is a trial number of factors and A^* corresponds to the minimum PRESS, and selecting the number of factors leading to a 75%-less probability that F > 1.

4.3. Quantitative results

In order to compare the performance of UPLS/RBL and MCR-ALS in terms of predictive ability when modeling second-order data, predictions were obtained on the eleven validation samples (Table 1). Prediction results corresponding to the application of MCR-ALS and U-PLS/RBL to the validation set showed relative error of prediction (REP%) values below 10% in all cases (Table 1). In order to appraise whether the recoveries were not statistically different than 100% a hypothesis test was applied. The experimental t_{exp} values were estimated following the Eq. (7)

$$t_{exp} = |100 - \overline{R}_{exp}| \frac{\sqrt{N}}{S_R} \tag{7}$$

where $\overline{R}_{exp}$ is the average experimental recovery and S_R the standard deviation of the recoveries. The recoveries are considering statistically different than 100% when t_{exp} value exceed the critical $t_{(\alpha,v)}$ value at

Table 3
Effect of constraints on rotational ambiguity for all components.

$f^{max}-f^{min}$	Constraints			
	1	1 and 2	1, 2 and 3	1, 2, 3 and 4
Atenolol	6.20	0.703	−0.0542	0.457
Enalapril	6.02	0.159	0.0164	−0.092
Amlodipine	1.35	0.267	0.0194	0.145
Gliclazide	6.40	0.365	−0.0348	0.049
Glibenclamide	12.8	0.508	0.0078	−0.013
Glimepiride	10.2	0.493	0.0176	0.066

Constraints: 1 normalization; 2 non–negativity: 3 unimodality; 4 trilineality.
$f^{max}-f^{min}$: corresponds to the difference between f^{max} and f^{min} values (see Ref. [21]).

Table 4
Figures of merit (LOD and LOQ) attained for U-PLS/RBL and MCR-ALS.

	LOD (ng mL^{-1})		LOQ (ng mL^{-1})	
	U-PLS/RBL	MCR-ALS	U-PLS/RBL	MCR-ALS
Atenolol	13.9	18.8	40.7	57.1
Enalapril	5.7	6.9	17.2	20.9
Amlodipine	16.8	28.3	50.4	85.6
Gliclazide	12.2	21.0	36.4	63.6
Glibenclamide	6.3	18.6	26.7	56.5
Glimepiride	9.4	18.1	28.2	54.9

level $\alpha, v = n - 1$ of freedom and n samples [22]. Considering 95% confidence level, the experimental t_{exp} value for all analytes in validation samples are lower than critical value $t_{(0.025,10)} = 2.228$, indicating that the recoveries are not statistically different than 100%.

LOD and LOQ were calculated according to Bauza [23]. The figures attained for U-PLS/RBL and MCR-ALS are displayed in Table 4. Interestingly, the LOQ values calculated are in the order of ng mL^{-1}, below 50 ng mL^{-1} for all the studied drugs, which allow quantitation in human serum in patients under treatment. In addition, these LODs and LOQs are lower than those reported by Georgita and Zhou [24,25], and even in the method previously developed by our group [5]. As can be seen U-PLS/RBL has better detection and quantification limits for all the studied drugs, however results obtained by MCR-ALS enable its usage as well, owing that differences cannot be considered as significant. On the other hand, the attained LODs and LOQs are lower than those obtained in previously published works by other authors, and

also compared with those obtained in our previous report [5]. But, it can be observed that in the first case not only a different extraction procedure was optimized and employed, but a different technique was implemented.

For recovery studies (accuracy), aliquots (250 μL) of serum sample were enriched with the six drugs in order to reach the concentration levels indicated in Table 2 and processed (see Section 3.5). Final solutions were injected into HPLC. Each sample was prepared by triplicate and evaluated by both strategies. The experimental t_{exp} value for all six drugs are lower than critical value $t_{(0.025,8)} = 2.306$, asserting the efficiency and the accuracy of the proposed method.

Fig. 2 shows the profiles retrieved by the MCR-ALS modeling of sample T1-1 (recovery study). As can be appreciated in this figure, severe overlapping occurs, especially in the time dimensions. Six components are present in the region R1 (Fig. 2A) and five components are present in the region R2 (Fig. 2C). MCR-ALS can differentiate between the calibration analytes and compounds of the matrix. Notably, the presence of three interferents is shown in the first region (Fig. 2A). On the other hand, only two interferents appear in the second region (Fig. 2C). Fig. 2B and D show the corresponding spectra of Regions 1 and 2, respectively. The extracted profiles match very well those corresponding to the pure compounds (not shown), indicating that the second-order advantage can be exploited.

In order to assess the method robustness, different chromatographic parameters were varied within a realistic range and the influence of these variables on recovery was studied. A twelve experiment Plackett–Burman design was built considering small variations in: mobile phase composition (% acetonitrile), chromatographic flow, and column temperature. Results (not shown) allowed us to conclude that the
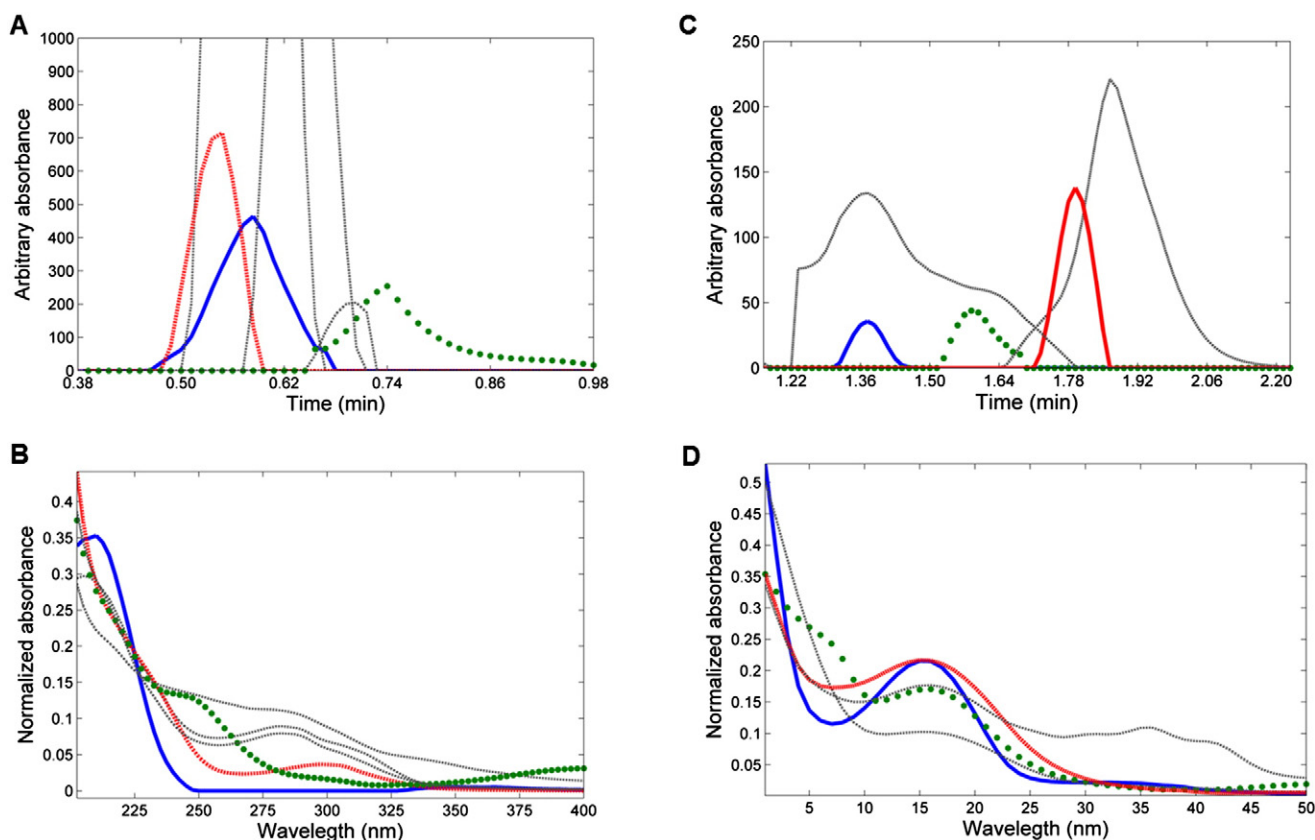
Fig. 2. Profiles retrieved by the MCR-ALS modeling of sample T1-1. (A) Three analytes and three interferents are present in Region 1. (C) Three analytes and two interferents are present in Region 2. (B and D) Spectra of Regions 1 and 2, respectively. Region 1 (antihypertensive drugs) atenolol red dashed line; enalapril blue solid line; amlodipine green dotted line; serum components grey lines. Region 2 (antidiabetic drugs) gliclazide blue solid line; glibenclamide green dotted line; glimepiride red dashed line; serum components grey line.

analytes can be correctly quantified even in presence of small variations of the main factors affecting the chromatographic procedure.

Table 5 shows the results obtained when analyzing thirty six samples from patients in treatment with the previous method developed by our group for the three antidiabetic drugs ("reference method") [5] and the method proposed herein. The elliptic joint confidence region (EJCR) test to evaluate the slopes and intercepts of the plots corresponding to predictions by the "reference method" vs. the studied one [26], was applied in order to carry out a comparison for each analyte (Fig. 3). In this case, it should be remarked that the analytes amlodipine, atenolol and enalapril could not be accurately quantified by use of the "reference method" owing to overlap of its peak with interference of the basal plasma, thus only antidiabetic drug predictions were compared through the EJCR method. As can be appreciated in Fig. 3, the elliptical domains contain the theoretically predicted value of the slope (1) and intercept (0). Fig. 3A shows the analysis of MCR-ALS predictions, while Fig. 3B corresponds to the analysis of U-PLS/RBL predictions. As can be seen in Fig. 3, better results are obtained using U-PLS/RBL than MCR-ALS for gliclazide. However, for the other analytes, the difference is not as notable. Therefore either algorithm can be used in the quantification of these drugs.

These observations indicate that both methods provide comparable results for glibenclamide, glimepiride and gliclazide. Interestingly, the new method provides better results than the total separation HPLC method, given lower quantification limits; this allows the quantification on samples 13, 25, 33 and 34. Furthermore, with this method we can quantify the presence of enalapril, amlodipine and atenolol. Run time was reduced by 50%. And last but not least, the reduction of solvents used reduces waste treatment and disposal costs, in accordance with green chemistry principles [27].

5. Conclusions

A liquid chromatographic method, based on modeling second-order data, was presented for the simultaneous determination of three antidiabetic drugs in human serum. This determination has been done in the presence of antihypertensive drugs by using second-order data generated by HPLC-DAD with both the multivariate curve resolution alternating least squares and the unfolded and multiway partial least-squares algorithms. The method was validated for all six analytes. Performance was excellent in terms of trueness and precision. In addition, a robustness study showed that the analytes can be accurately quantified even in presence of small variations of the main factors affecting the chromatographic procedure. The proposed strategy considerably reduces the complexity of the resolution and in consequence the processing time. By use of this method, amounts of solvents used in the separation step are reduced, which is highly recommended by the principles of green analytical chemistry.

Table 5
Analysis of real samples.

Sample	Atenolol		Enalapril		Amlodipine		Gliclazide			Glibenclamide			Glimepiride		
	Anal. A[a]	Anal. B[b]	Anal. A[a]	Anal. B[b]	Anal. A[a]	Anal. B[b]	Ref[c]	Anal. A[a]	Anal. B[b]	Ref[c]	Anal. A[a]	Anal. B[b]	Ref[c]	Anal. A[a]	Anal. B[b]
M1	0.251	0.246					0.473	0.459	0.469						
M2										0.151	0.150	0.153			
M3			0.192	0.189						0.290	0.265	0.279			
M4			0.362	0.375									0.229	0.213	0.218
M5					0.065	0.068							0.282	0.286	0.290
M6	0.155	0.158											0.245	0.245	0.242
M7							0.383	0.386	0.380						
M8													0.255	0.253	0.257
M9			0.272	0.267									0.280	0.285	0.284
M10							0.736	0.737	0.740						
M11	0.718	0.721					ND	0.116	0.119						
M12			0.315	0.308			ND	0.110	0.109						
M13													ND	0.117	0.106
M14	0.419	0.423								0.271	0.278	0.276			
M15							0.614	0.618	0.595						
M16										0.211	0.223	0.213			
M17			0.130	0.133									0.261	0.254	0.277
M18										0.185	0.189	0.181			
M19	0.215	0.210					1.424	1.427	1.417						
M20	0.178	0.173								0.522	0.537	0.562			
M21			0.205	0.195						0.238	0.247	0.222			
M22			0.375	0.388									0.364	0.354	0.376
M23							0.261	0.265	0.255						
M24					0.102	0.108	0.327	0.319	0.327						
M25	0.216	0.205											ND	0.110	0.114
M26							0.359	0.336	0.348						
M27										0.405	0.446	0.402			
M28			0.302	0.298						0.151	0.156	0.149			
M29	0.199	0.197											0.219	0.223	0.217
M30							0.464	0.460	0.460						
M31	0.463	0.458					1.080	1.098	0.902						
M32					0.075	0.073				0.134	0.124	0.134			
M33										ND	0.106	0.117			
M34										ND	0.131	0.138			
M35			0.118	0.115									0.228	0.215	0.219
M36			0.122	0.122									0.304	0.304	0.301
Slope								1.016	0.934		1.066	1.075		1.008	1.110
Intercept								−0.011	0.017		−0.011	−0.018		−0.006	−0.027

ND: not quantifiable.

[a] Concentration are given in μg mL^{-1}. Anal. A: U-PLS/RBL.
[b] Anal. B: MCR-ALS.
[c] Reference method.
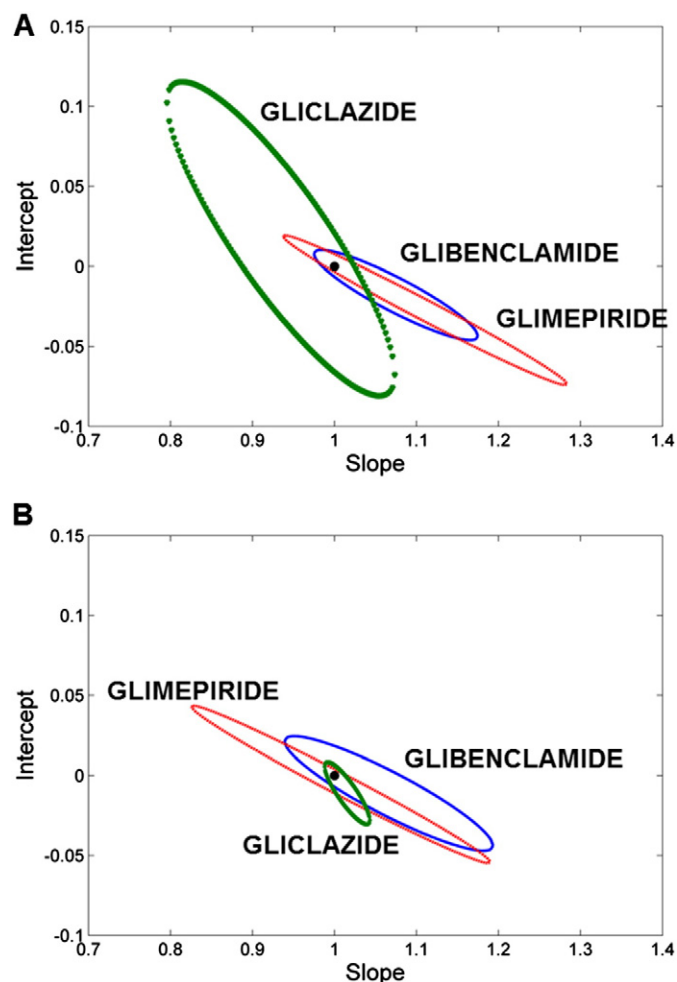
Fig. 3. Elliptic joint confidence region (EJCR) for the predictions of glibenclamide, glimepiride and gliclazide. (A) Ellipses obtained by comparing results processed by MCR-ALS and reference method. (B) Analysis of U-PLS/RBL and reference method predictions. Gliclazide: green line. Glibenclamide: blue line Glimepiride: red line.

Acknowledgments

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